11-1-2017

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HHS Public Access

Author manuscript

Psychoneuroendocrinology. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

Psychoneuroendocrinology. 2016 November ; 73: 252–262. doi:10.1016/j.psyneuen.2016.08.017.

Behavioral, Pharmacological and Neuroanatomical Analysis of Serotonin 2C Receptor Agonism on Maternal Behavior in Rats

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Summary

As a highly motivated social behavior, maternal behavior in rats has been routinely used to study psychoactive drugs for clinical, neuroscience and pharmacological purposes. Recent evidence indicates that acute activation of serotonin 2C (5-HT_{2C}) receptors causes a disruption of rat maternal behavior. The present study was designed to elucidate the behavioral, pharmacological mechanisms and neuroanatomical basis of this 5-HT_{2C} effect. First, we replicated the finding that acute MK212 injection (2.0 mg/kg, a highly selective 5-HT_{2C} agonist) disrupts maternal behavior, especially on pup retrieval. Interestingly, this disruption was significantly attenuated by 4-h pup separation (a procedure putatively increased maternal motivation). MK212 also suppressed food retrieval, indicating that it has a general effect on motivated behaviors. Second, we showed that MK212 disrupts maternal behavior by specifically activating 5-HT_{2C} receptor, as pretreatment with a 5-HT_{2C} receptor antagonist SB242084 (0.6 and 1.0 mg/kg) alleviated MK212-induced disruption on pup retrieval. Third, we microinjected MK212 into various brain regions implicated in the regulation of maternal behavior: nucleus accumbens shell (25, 75, 250 ng/0.5μl/side), medial prefrontal cortex (25 and 250 ng, 1, 2 and 5 μg/0.5μl/side), and medial preoptic area (MPOA, 75 ng, 1 and 5 μg/0.5μl/side). Pup retrieval and other maternal responses were not affected by any of these manipulations. Finally, we used c-Fos immunohistochemistry to identify the central mechanisms of the acute and repeated MK212 effects on maternal behavior. Acute MK212 (2.0 mg/kg) disrupted pup retrieval and concurrently decreased c-Fos expression in the ventral part of lateral septal nucleus (LSv), MPOA, dentate gyrus (DG) and dorsal raphe (DR), but increased it in the central amygdala (CeA). Five days of repeated MK212 (2.0 mg/kg) treatment produced a

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Conflicts of interest

All the authors declare that they have no conflict of interest.

Contributors.

Dr. Ming Li designed and supervised all experiments, and also contributed to the editing of the manuscript. Dr. Ruiyong Wu participated in the first experiment and wrote the first draft of this manuscript. Dr. Jun Gao and Shinnyi Chou participated in the third and forth experiments, respectively. Mr. Davis participated in the second experiment and contributed to all the behavioral analysis.

persistent disruption of pup retrieval and only decreased c-Fos expression in the DR. These findings not only confirm a role of 5-HT_{2C} receptor in rat maternal behavior, but also suggest that the coordinated 5-HT_{2C} activity in various limbic (e.g., LSv, DG, CeA), hypothalamic regions (e.g., MPOA) and brainstem areas (e.g. DR), is likely involved in the mediation of important psychological processes (e.g. motor function, motivation) necessary for the normal expression of maternal behavior.

Keywords

Serotonin 2C receptor; MK212; SB242084; Maternal behavior; Maternal motivation; Pup retrieval

1. Introduction

Maternal behavior in rats provides a valuable social interaction model with high ecological validity for the study of the neurobiological mechanisms of mothering and psychoactive drugs in a social domain (Fleming and Corter, 1988; Fleming and Corter, 1995; Li, 2015). Much research on the neurochemical basis of maternal behavior has been focusing on the mesolimbic and mesocortical dopamine (DA) systems (Afonso et al., 2007; Febo et al., 2010; Hansen et al., 1991; Keer and Stern, 1999; Li and Fleming, 2003a, b; Numan, 2007; Numan et al., 2005). For example, both dopamine D_1 and D_2 receptors in the nucleus accumbens and medial preoptic area (MPOA) are involved in the regulation of maternal behavior (Numan et al., 2005; Stolzenberg et al., 2007; Stolzenberg et al., 2010), as activation or blockade of either receptor by psychoactive drugs (e.g. apormorphine and haloperidol) impairs maternal response in rats (Giordano et al., 1990; Keer and Stern, 1999; Silva et al., 2001).

Recent work suggests that serotonin, via its actions on 5-HT_{2A} and 5-HT_{2C} receptors, also plays an important role in the mediation of maternal behavior in rats. First, atypical antipsychotic drugs (e.g. clozapine) which possess potent antagonist actions against 5-$HT_{2A/2C}$ receptors, dose-dependently disrupt active components of maternal behavior, such as pup retrieval, pup licking and nest building (Li et al., 2005; Li et al., 2004). Second, acute administration of 2,5-dimethoxy-4-iodo-amphetamine (DOI, a selective 5-$HT_{2A/2C}$ agonist) disrupts maternal performance, whereas pretreat of DOI dose-dependently reverses the clozapine (CLZ)-induced maternal disruption, suggesting that both activation and blockade of 5-HT_{2A} and/or 5-HT_{2C} receptors affect maternal behavior (Zhao and Li, 2009a, 2010). Finally, acute systemic injection of MK212, a highly selective 5-HT_{2C} receptor agonist, also disrupts active components of rat maternal behavior in a dose-dependent fashion (Chen et al., 2014). Overall, it is clear that 5-HT_{2C} receptor plays a regulatory role in maternal behavior, although much of the evidence comes from acute pharmacological studies and the specific mechanisms at various levels have not been elucidated.

The present study was designed to investigate the behavioral, pharmacological, and neuroanatomical mechanisms underlying the 5-HT_{2C} receptor action in maternal behavior. Behaviorally, we employed two techniques. One was pup separation, a procedure putatively increasing maternal motivation. Removing pups from dams for several hours (> 3 h) prior to

maternal behavior testing is shown to restore pup retrieval deficits induced by massive dopamine depletion (Hansen, 1994), or reverse CLZ-induced disruption on maternal performance (Zhao and Li, 2009b). We treated our subjects with MK212 and tested them under the pup-separation (4 h) and no-pup-separation conditions, respectively. If pup-separation is able to antagonize the effect of MK212, it would suggest that at least some aspect of the MK212's disruption was due to its effect on animals' motivation. The second technique was food-hoarding test, as a nonmaternal motivated behavior to compare and determine the nature of observed pup retrieval deficits (Numan, 1990; Numan and Corodimas, 1985). Because pup and food retrieval share some basic psychological processes, such as oral sensorimotor, incentive motivation, and sequential organization of motor acts (Whishaw et al., 1990), if MK212 also disrupts food retrieval, then 5-HT_{2C} receptor might be broadly involved in motivated behaviors and its role is not maternally specific. Pharmacologically, we used a highly selective 5-HT_{2C} receptor antagonist SB242084 (Kennett et al., 1997) and determined the receptor specific effect of MK212. Neuroanatomically, we also employed two approaches. One was the microinjection. MK212 was microinjected into various brain regions implicated in the regulation of maternal behavior, such as the nucleus accumbens shell (NAs) (Li and Fleming, 2003a, b), medial prefrontal cortex (mPFC) (Afonso et al., 2007; Febo et al., 2010), and MPOA (Numan et al., 1977; Stack et al., 2002) and maternal behavior was tested using a within-subjects design. The second approach was the c-Fos immunohistochemistry. We examined both acute and repeated treatment effects of MK212 (2.0 mg/kg) on c-Fos expression in various brain regions (NAs, mPFC and dorsal raphe, etc.).

2. Materials and methods

2.1. Animals

Naïve pregnant female Sprague-Dawley rats (gestational days 6 upon arrival to the animal facility) were purchased from Charles River Inc. All rats were housed individually in 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages under 12-h light/dark conditions (lights on at 6:30 am), and had access to standard laboratory rat chow and tap water *ad libitum*. The colony was maintained with a controlled temperature (21 ± 1 °C) and a relative humidity of 45–60%. Experiments were conducted during the light cycle. All animal manipulations were reviewed and approved by the University of Nebraska Institutional Animal Care and Use Committee, and were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs and choices of dosage

MK212 [6-Chloro-2-(l-piperazinyl) pyrazine hydrochloride] and SB242084 [6-Chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridiny]-1H-indole-1-carboxyamide dihydrochloride hydrate] were obtained from Tocris Bioscience (Ellisville, MO, USA). MK212 was dissolved in 0.9% saline and administrated subcutaneously at 2.0 mg/kg except central infusion study in Experiment 3. This dose of MK212 produces a reliable disruption on maternal behavior (Chen et al., 2014). For the central infusion, various doses of MK212 (primarily based on relevant published studies) were microinjected into different brain regions (Filip and Cunningham, 2002, 2003; Pentkowski et al., 2010): 25 ng, 75 ng, 250 ng/

0.5μl/side for NAs; 75 ng, 1.0 μg, 5.0 μg/0.5μl/side for MPOA; 25 ng, 250ng, 1 μg, 2μg, 5μg/0.5 μl/side for mPFC. The bilateral microinjection (0.5 μl at 0.5μl/min) started 1 min after the insertion of the injector, which remained in place for an additional 1 min before removal to allow for drug diffusion. In present study, 0.9% saline was used as vehicle for MK212. SB242084 was dissolved in 30% dimethyl sulfoxide (DMSO) mixed with saline (VEH-1) and injected subcutaneously at the doses of 0.6 and 1.0 mg/kg. These doses are in the range of effective doses (0.2 mg/kg to 1.0 mg/kg) found in several recent reports (Boulougouris et al., 2008; Burghardt et al., 2007; Strong et al., 2009).

2.3. Basic experimental procedure and maternal behavior test

The basic procedure was identical to what has been described in our previous studies (Chen et al., 2014; Zhao and Li, 2009b). Starting 2 or 3 days prior to the first possible expected parturition date, the subjects were monitored every morning and afternoon for signs of parturition. Once the dam was found with pups in the morning (that day was designated as postpartum day 1, PP1) or in the afternoon (PP 0), two shredded paper towels were provided for nesting materials. On PP 2, each litter was culled to 8 pups (4 males and 4 females with the most visible milk bands) and all subjects were changed to clean observation cages with their litter.

Maternal behavior test was initiated by taking the eight pups away from the mother and destroying the nest. Ten seconds later, the pups were placed in the corner of the cage diagonal to the nest site or dam sleeping corner. Each test was recorded by video cameras and analyzed manually using a laptop computer with an event-recording program (JWatcher, http://www.jwatcher.ucla.edu). The raters were blind to each subject's drug condition. The following behaviors were recorded and analyzed: pup retrieval (a rat picking up a pup in her mouth and carrying it back to the nest site), pup nursing (a rat positioning herself over the pups with legs splayed to accommodate the pups, including hover, high and low crouching-over posture), pup licking (female rat placing its tongue on the anogenital area and the rest of a pup's body), nest building (a rat picking up nest material in her mouth and transporting it back to the nest site or pushing the material with her forepaws towards the nest site). The first pup retrieval latency was defined as the time elapsed from the first pup approach to the retrieval of the first pup into the nest. 600s was assigned to non-responders who did not approach or retrieve the testing pups. After the test, unretrieved pups were returned to the nest site. On PP 2 or 3, to screen for baseline maternal performance and habituate dams to the maternal behavioral testing procedure, we conducted one maternal behavior test (removing pups then return them 10 s later), which was not video-recorded nor analyzed.

2.4. Experiment 1: Effect of pup separation on MK212-induced maternal disruption of pup retrieval: A comparison with food retrieval test

In this experiment, two groups of postpartum rats (MK212-2.0mg/kg, n = 16; VEH, n = 15) were used. A series of maternal behavior tests were conducted on PP 4 and 6. On each test day, maternal behavior was observed for 10 min at 4 time points, with the first one at 0.5 h before the drug injections (i.e., baseline), and the rest being carried out at 0.5, 2 and 4 h after the injections. These test time points were chosen primarily based on our and others' MK212 studies (Chen et al., 2014; de Mello Cruz et al., 2005). We performed a cross-over

design. On PP 4, mother rats were allocated at random to receive either MK212 (n = 8) or VEH treatment (n = 7) and tested under the pup-separation (PS) condition in which their pups were taken away from them 4 h before the baseline test (0.5 h before the drug injection) on that day. Pups were removed from the mothers and placed into a bowl with nesting material on a temperature-controlled heating pad (34°C). The other dams (n = 8 for MK212; n = 8 for VEH) were tested without separation from their pups (no-pup-separation condition, NS). On PP 6, the same procedure was applied, but the rats tested in the pup-separation condition on Day 4 were tested in the no-pup-separation condition, and ones previously tested in the no-pup-separation condition were tested in the pup-separation condition (a complete counterbalanced design).

This cross-over design allowed us to consecutively test each subject which received two experimental manipulations during the course of the experiment. Various measures of maternal behavior were recorded (see below for testing details). The food hoarding tests were conducted on PP 8 and 10. Two testing conditions were used: 6-h food-deprivation and no-food-deprivation. The basic procedure was adopted from previous studies (Numan and Corodimas, 1985; Whishaw et al., 1990). Before each test, pups were taken away and nests were destroyed. Ten seconds later, eight Supreme Mini-Treats™ Chocolate Flavor food pellets were placed in the front of the cage. Video recording started immediately after the placement of pellets for 10 min. At the end of each test, unretrieved and unconsumed pellets were removed, and the pups were returned to the dams. Number of retrieved pellets and retrieval latency was recorded.

2.5. Experiment 2: Receptor specificity of MK212's disruptive effect on pup retrieval

In this study, we tested 7 postpartum rats using a within-subjects Latin square design to see if pretreatment of SB242084 (0.6 and 1.0 mg/kg) could attenuate the disruptive effect of MK212 (2.0 mg/kg) on pup retrieval. These doses of SB242084 were chosen based on several recent reports (Boulougouris et al., 2008; Burghardt et al., 2007; Strong et al., 2009). In addition, our own pilot study did not find any pup retrieval disruption by acute injection of SB242084 at 0.2, 0.6 and 1.0 mg/kg (unpublished data). Thus, any reversal effect of SB242084 could only be attributed to its counteraction again MK212. On PP 4, 6, 8, and 10, mother rats were randomly assigned to receive either a double injection of VEH-1 + VEH, VEH-1 + MK212, SB242084-0.6 + MK212, or SB242084-1.0 + MK212. All rats were tested under all the treatment conditions on every other day, so they served as their own controls. VEH-1 or SB242084 was injected 10 min before the MK212. Maternal behavior was tested for 10 min at 30 min before, 30 min, 60 min and 24 h after MK212 injection. Number of pups retrieved during the 10 min test period was used as the major index of MK212 effect and the reversal effect of SB242084.

2.6. Experiment 3: Neural basis of MK212's effects on maternal behavior: a microinjection study

In this experiment, we attempted to identify the brain sites where MK212 acts to disrupt maternal behavior by centrally infusing MK212 into the NAs, MPOA or mPFC, three possible sites implicated in the action of MK212 (de Almeida et al., 2006; Liu et al., 2007; Pentkowski et al., 2010). On day 11–13 of gestation, rats were anaesthetized using a mixture

of ketamine HCl (90 mg/kg) and xylazine (4 mg/kg) (ip), and implanted with bilateral stainless-steel guide cannulas (22 gauge; Plastics One, Inc.) into the NAs (n = 6), MPOA (n = 7) or mPFC (n = 12). The incisor bar was set at −3.4 mm. The stereotaxic coordinates for the NAs were: anteroposterior (AP) +1.5 mm, mediolateral (ML) ±1.0 mm, dorsoventral (DV) −5.5 mm (Reynolds and Berridge, 2003). For the MPOA cannulation, the coordinates were set as: AP −0.5 mm, ML ± 0.75 mm, DV −6.5 mm (Guarraci et al., 2004; Small et al., 2003). For the mPFC, the coordinates were: AP + 3.0 mm, ML ± 0.75 mm, DV −2.2 mm (Febo et al., 2010; Xie and Steketee, 2009).

Using a within-subjects Latin square design, 6 postpartum females rats were tested at 10 min after central infusion of VEH, MK 212 at 25, 75, or 250 ng/0.5μl/side on PP3, 5, 7, and 9 for the NAs study. For the MPOA, 7 rats were tested at 10 and 60 min after intra-MPOA infusion of VEH, 75 ng, 1.0 μg, or 5.0 μg/0.5μl/side of MK212 on PP 4, 6, 8 and 10, respectively. For the mPFC, two batches of 6 rats were tested under a lower (VEH, 25 ng, 250 ng, 1.0 μg/0.5μl/side) and a higher dose range (VEH, 25 ng, 2.0 μg, 5.0 μg/0.5μl/side) using the same design as in the MPOA experiment. At the end of behavioral tests, rats were sacrificed and perfused. Their brains were sectioned and then stained with cresyl violet before viewing cannula placement as previous report (Feng et al., 2015). The location of the injection site was mapped onto a stereotaxic atlas (Paxinos, 2005) (Figure 1).

2.7. Experiment 4: Neural basis of MK212's effects on maternal behavior: a c-Fos immunohistochemistry study

A total of 23 postpartum rats were randomly divided into one of four groups: repeated VEH (n = 6), repeated 2.0 mg/kg MK212 (n = 6), acute VEH (n = 5) and acute 2.0 mg/kg MK212 (n = 6) group. This dose was chosen based on our previous work showing that acute injection of MK212 dose-dependently disrupts rat maternal performance with 2.0 mg/kg MK212 having the most disruption (Chen et al., 2014). For the repeated groups, maternal behavior was tested for 10 min once daily from PP 6 to 9 starting at 30 min after MK212 or VEH injection. For the acute groups, maternal behavior was only tested during PP 6 to 9 and no injection was done.

On PP 10, 1 h after the MK212 or VEH injection, all rats were deeply anesthetized and perfused as described in our previous work, and their brains were extracted for c-Fos immunoreactivity staining (Zhao and Li, 2010, 2012). Briefly, coronal sections were incubated with a rabbit polyclonal anti-c-Fos antibody (Ab-5, 1:20000 dilution, Calbiochem, CA, USA) for 48 h at 4°C. Sections were then incubated with a biotinylated goat anti-rabbit secondary antibody (1:200 dilution, Vector Laboratories, Burlingame, CA, USA) in PBS containing 1% normal goat serum for 2 h at RT. They were processed with avidin-biotin horseradish peroxidase complex (1:200 dilution, Vectastain Elite ABC Kit, Vector Laboratories). The immunoreaction was visualized with peroxidase substrate (DAB Substrate Kit for Peroxidase, Vector Laboratories). After staining, sections were mounted on gelatin-coated slides, air-dried, dehydrated and coverslipped. As a control, the primary antibody was substituted with normal goat serum. No corresponding nucleus or cytoplasm was immunostained in the control.

Microscopic images were captured with a digital camera (INFINITY lite, Canada) furnished with an Olympus CX41RF microscope (Japan) using × 10 objective lens. The number of positive cells characterized by clearly labeled nuclei was counted unilaterally in six serial sections with comparable anatomical levels across the treatment groups. We focused on the NAs, mPFC, MPOA, dorsolateral striatum (DLSt), ventral part of lateral septal nucleus (LSv), central amygdala (CeA), ventral tegmental area (VTA), and dorsal raphe (DR), because 5-$HT_{2A/2C}$ receptor agonist (DOI) and antagonists (CLZ and olanzapine, OLZ) are shown to have effects on these regions in mother rats (Zhao and Li, 2010, 2012). DR was also chosen because it is a major serotonergic brain site (Azmitia and Segal, 1978; Liu et al., 2000; Queree et al., 2009; Steinbusch, 1981). Other brain regions analyzed included the ventral bed nucleus of the stria terminalis (vBNST), medial amygdala (MeA), dentate gyrus (DG), and periaqueductal gray (PAG). The levels of brain slices were: Bregma 3.00 mm for mPFC, 1.92 mm for the NA and DLSt, 1.44 mm for the LSv, −0.24 for MPOA and vBNST, −2.92 mm for CeA, MeA and DG, −6.24 mm for VTA, and −7.92 mm for the PAG and DR according to Paxinos and Watson (Paxinos, 2005). With the help of ImageJ software, cell counts were made within a 1.8 × 1.8 mm^2 unit area of each region of interest by an experimenter blind to the treatment condition. In a given area from distinct treatments, the images were threshold to the same value by means of eliminating background noise staining to ensure that the positive cells were selected. The number of cells in a given brain region from unilateral sites per rat were averaged. The values from each treatment group were averaged to obtain the final mean ± SEM.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). To examine the pup-separation and food-deprivation effects, data from the PP 4 and 6, as well as PP 8 and 10 under the same testing condition (e.g. pup-separation on PP4 and 6, food-deprivation on PP8 and 10) were combined and analyzed using repeated measures analysis of variance (AVOVA). Group differences at different test time points were further investigated using simple main effect tests (one-way ANOVA) followed by LSD post hoc tests for multiple comparisons where appropriate. Data from the experiments with a Latin square design were analyzed using Paired-Sample test. Repeated measures ANOVAs were conducted to examine the effects of repeated MK212 administration from PP 6 to 9. c-Fos Data were analyzed using multivariate analysis of variance in a 2×2 design (drug and treatment condition as between-subjects factors, brain regions as within-subjects factors), and significant effects were followed up using the LSD post hoc test comparing the within-treatment condition across drug, and within-drug condition across treatment. Because the latency was not normally distributed, those data were analyzed using nonparametric Kruskal-Wallis test, and Mann-Whitney U test if the overall significant effects were determined. All data are presented as mean ± SEM. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Experiment 1: Effect of pup separation on MK212-induced maternal disruption of pup retrieval: A comparison with food retrieval test

In agreement with our previous report (Chen et al., 2014), acute MK212 injection severely disrupted various active components of maternal behavior. In comparison to the VEH groups, rats treated with MK212 retrieved significantly fewer pups (all $p < 0.001$) and took a much longer time to complete pup retrievals (all $p < 0.001$) at the 30 min post injection testing point under both the pup separation and no separation conditions (Figure 2A and B). In addition, they also spent less time on pup nursing (all $p < 0.001$), licking (no-pup-separation: $p = 0.107$; pup-separation: $p = 0.001$) and nest building (all $p < 0.001$) (Figure 2C–E). Furthermore, pup separation was effective in increasing the amount of time spent by the mothers on nursing (all $p < 0.001$) and licking (VEH: $p = 0.078$; MK212: $p = 0.003$) and decreasing nest building (VEH: $p = 0.051$) at the baseline point, indicating the effectiveness of this procedure to increase maternal performance, possibly by increasing maternal motivation. More importantly, it significantly mitigated the MK212-induced pup retrieval deficit at the 30 min point, as evidenced by the increased number of pups retrieved ($p = 0.017$) (Figure 2A) and decreased pup retrieval latency ($p = 0.009$) (Figure 2B) compared to the no-pup-separation + MK212 group.

At the baseline point, repeated measures ANOVA revealed a main effect of no-pup-separation/pup-separation on pup nursing [$F(1, 58) = 107.717$, $p < 0.001$], licking [$F(1, 58) = 12.829$, $p = 0.001$], and nest building [$F(1, 58) = 3.898$, $p = 0.053$]. At the 30 min testing point, there was a significant main effect of drug treatment on pup retrieval [number of pups retrieved: $F(1, 58) = 113.679$, $p < 0.001$; latency of first pup retrieval: $F(1, 58) = 123.774$, $p < 0.001$], nursing [$F(1, 58) = 113.929$, p < 0.001], pup licking [$F(1, 58) = 13.275$, $p = 0.001$], and nest building [$F(1, 58) = 43.52$, $p < 0.001$]; the interaction between two factors was also significant for pup retrieval [number of pups retrieved: $F(1, 58) = 4.104$, $p = 0.047$; latency of first pup retrieval: $F(1, 58) = 4.808$, $p = 0.032$]. There was no significant main effect of two factors or interaction between them on these parameters at 2 and 4 h testing points.

Acute MK212 treatment also disrupted food retrieval. The MK212-treated rats retrieved fewer chocolate flavored food pellets (all $p < 0.001$) and took a much longer time to retrieve them into the nest site (all $p < 0.001$) than the VEH rats at the 30 min, 2 h and 4 h testing points under both food-deprivation and no-food-deprivation conditions (Figure 3A and B). Six-hour food deprivation failed to alleviate these deficits (all $p > 0.05$) (Figure 3A and B), as there was no significant improvement in the number of food retrieved and first food retrieval latency. Repeated measures ANOVA revealed a significant main effect of drug treatment on the number of food retrieved [$F(1, 58) = 89.458$, $p < 0.001$ at 30 min; $F(1, 58) = 103.053$, $p < 0.001$ at 2 h; $F(1, 58) = 31.072$, $p < 0.001$ at 4 h] and latency of first food retrieval [$F(1, 58) = 190.662$, $p < 0.001$ at 30 min; $F(1, 58) = 268.338$, $p < 0.001$ at 2 h; $F(1, 58) = 25.249$, $p < 0.001$ at 4 h], but no significant effect of no-food-deprivation/food-deprivation or interaction between the two factors.

3.2. Experiment 2: Receptor specificity of MK212's disruptive effect on pup retrieval

Pretreatment of SB242084 significantly improved the MK212-induced pup retrieval deficits. In comparison to the VEH-1 + VEH condition, rats under the VEH-1 + MK212 retrieved fewer pups into the nest at the 30 min ($p = 0.014$) and 60 min ($p = 0.014$) points. Pup retrieval improved only under the SB242084-0.6+MK212 ($p = 0.046$ at 30 min; $p = 0.083$ at 60 min) conditions (Figure 4), but the differences between the SB242084-0.6/1.0 + MK212 and VEH-1 + VEH were not significant (all $p > 0.05$ at 30 min and 60 min), indicating a relatively complete reversal.

3.3. Experiment 3: Neural basis of MK212's effects on maternal behavior: a microinjection study

To examine the neuroanatomical basis of action of MK212 in maternal behavior, we microinjected MK212 at 25, 75, or 250 ng/side into the NAs. All the injection sites were verified in the intended targeted areas. Results showed that intra-NAs infusion of MK212 had no effect on pup retrieval (all $p > 0.05$) (Figure 5A), as well as other maternal responses (e.g. pup licking, nursing and nest-building, data not shown). Similarly, intra-MPOA infusion of M212 at 75 ng, 1.0, and 5.0 μg/side also did not affect maternal performance. There was no significant difference among the four drug treatment conditions (all $p > 0.05$) (Figure 5B). Finally, both batches of dams with intra-mPFC injections of two dose ranges (batch 1: 25, 250 ng or 1 μg/side; batch 2: 25 ng, 2 or 5 μg/side) also did not show any difference in the number of pup retrievals and other maternal responses under any treatment condition (all $p > 0.05$) (Figure 5C).

3.4. Experiment 4: Neural basis of MK212's effects on maternal behavior: a c-Fos immunohistochemistry study

Failure to identify any specific brain region that may mediate the MK212 effect on maternal behavior prompted us to use the c-Fos immunohistochemistry technique to map out a broad neural network. Behaviorally, we replicated the acute disruptive effect of MK212 on pup retrieval. In addition, we found that repeated MK212 treatment produced a persistent disruption of pup retrieval (all $p < 0.05$) (Figure 6A), as repeated measures ANOVA showed a significant main effect of drug treatment [$F(1, 10) = 39.205$, $p < 0.001$] on the number of pup retrieval across the 4 test days.

In comparison to the VEH treatment, acute MK212 treatment significantly decreased c-Fos immunoreactivity in the LSv ($p = 0.025$), MPOA ($p = 0.035$), DG ($p = 0.007$) and DR ($p = 0.011$), but increased it in the CeA ($p = 0.029$). Repeated MK212 treatment only decreased c-Fos immunoreactivity in the DR ($p = 0.021$) (Figures 6B and C). Comparisons between acute and repeated MK212 treatments showed that repeated MK212 rats had significantly lower c-Fos immunoreactivity in the LSv ($p = 0.017$) and CeA ($p = 0.011$) than acute MK212 rats. Two-way ANOVA revealed a main effect of drug treatment on c-Fos immunoreactivity in the LSv [$F(1, 19) = 20.642$, $p = 0.001$], DG [$F(1, 19) = 18.691$, $p = 0.001$], CeA [$F(1, 19) = 13.512$, $p = 0.003$], and DR [$F(1, 19) = 15.679$, $p = 0.002$], a main effect of acute/repeated treatment in the LSv [$F(1, 19) = 13.465$, $p = 0.003$], MPOA [$F(1, 19) = 4.802$, $p = 0.049$] and VTA [$F(1, 19) = 7.371$, $p = 0.019$], and a significant interaction between the two factors in the MPOA [$F(1, 19) = 5.257$, $p = 0.041$], DG [$F(1, 19) = 6.484$,

$p = 0.026$], CeA [$F(1, 19) = 7.789$, $p = 0.016$] and VTA [$F(1, 19) = 5.466$, $p = 0.038$]. Interestingly, both acute and repeated MK212 did not affect c-Fos immunoreactivity in the mPFC, NAs, NAc, vBNST and MeA, brain regions previously implicated in the regulation of rat maternal behavior (Afonso et al., 2007; Brunton and Russell, 2008; Li and Fleming, 2003b).

4. Discussion

The present study provided a comprehensive evaluation of various mechanisms of 5-HT_{2C} agonism on maternal behavior. Specifically, we found that maternal behavior, especially pup retrieval, was severely disrupted at the 30 min testing point after MK212 injection (2.0 mg/kg) and this disruption was attenuated to some extent by 4-h pup separation. In addition, acute MK212 treatment also significantly disrupted food retrieval at the 30 min, 2 h and 4 h time points after injection, and 6-h food deprivation was too weak to reverse this effect of MK212. These findings suggest that MK212 may have a general suppressive effect on motivated behaviors. Next, we showed that pretreatment of the 5-HT_{2C} receptor antagonist SB242084 alleviated MK212-induced disruption in pup retrieval, confirming that MK212 disrupts maternal behavior mainly through activating 5-HT_{2C} receptors. Third, we failed to use the microinjection technique to identify specific brain sites in which 5-HT_{2C} receptor exerts its maternal effect, as microinjection of a wide range of MK212 doses into the NAs, MPOA or mPFC did not cause any disruption in pup retrieval. However, the c-Fos immunohistochemistry technique was able to show that the 5-HT_{2C} receptor in a neural network involving LSv, DG, CeA, MPOA, and DR is likely to play a role in maternal behavior, as acute MK212 altered c-Fos expression in the LSv, MPOA, CeA, DG and DR; and repeated MK212 further decreased c-Fos expression in the DR.

Chen et al. (2014) first reported that acute MK212 administration disrupts maternal responses in rats, especially for pup retrieval. Because pup retrieval is a proactive behavior of the dam and is usually used as an indicator of maternal motivation (Levy and Keller, 2009), we speculated that MK212 may disrupt pup retrieval by suppressing maternal motivation. The findings that activation of 5-HT_{2C} receptors can decrease dopamine release in the NAs and cell firing in the VTA are also consistent with this speculation (Di Giovanni et al., 2006; Di Matteo et al., 2002), as the mesolimbic dopamine system is known for its role in motivation and maternal behavior (Li and Fleming, 2003b; Numan, 2007) and other evidence suggests that 5-HT_{2C} receptor agonism could decrease various motivated behaviors (Grauer et al., 2009; Higgins et al., 2012). To test this idea, we employed a pup-separation technique, which has been used to restore pup retrieval deficit induced by 6-OHDA lesions in the ventral striatum (Hansen, 1994). Four hours of pup-separation prior to the maternal behavior tests significantly attenuated the MK212-induced decrease in pup retrieval and shortened pup retrieval latency, without mitigation on other active maternal responses. This finding indicates that MK212 may disrupt maternal behavior by partially suppressing mothers' motivation to take care of the young. It should be noted that because pup separation failed to completely reverse MK212-induced maternal deficits to the vehicle level, other behavioral effects of MK212 may also contribute to its maternal effect, such as its sedative effect or its motor suppression effect, as MK212 does induce hypolocomotion in rodents (Lucki et al., 1989; Stiedl et al., 2007). In addition, rats treated with MK212 were

not tested immediately after the 4-h separation (i.e., they had been with their mothers for about 1 h before the 1st MK212 test), and the 1-h reunion right after the 4-h pup separation might have attenuated the separation-induced increase in maternal motivation, leading to a reduced reversal. This might explain well why the pup separation employed in this study was not as effective as the one used in our previous study (Zhao and Li, 2009b), which did make the drug test coincided with the end of 4-h pup separation. At this moment, we could only conclude that there might be multiple behavioral mechanisms involved in the maternal disruptive effect of MK212.

Another way to study the potential effect of MK212 on maternal motivation is to compare its effect on other motivated behaviors. In this study, we used a food-hoarding test (Whishaw et al., 1990), which is similar to pup retrieval in terms of the required oral sensorimotor, incentive motivation, and sequential organization of motor acts. This task was chosen also because 5-HT_{2C} receptors are well documented to be involved in the regulation of feeding behavior and food intake. Treatment with 5-$HT_{1B/2C}$ receptor agonist 1-(*m*-chlorophenyl) Piperazine (*m*CPP) produces behaviorally selective reductions in food intake in rats (Kitchener and Dourish, 1994), mice (Hewitt et al., 2002), and human (Sargent et al., 1997). The effects of *m*CPP on feeding in mice are also attenuated by SB242084 (Hewitt et al., 2002). Selective 5-HT_{2C} receptors agonists, including Ro 60-0175 and MK212, reduce feeding behavior (Clifton et al., 2000; Fletcher et al., 2009; Somerville et al., 2007; Vickers et al., 2000), while the 5-HT_{2C} receptor antagonist RS100011 enhances food intake in rats (Bonhaus et al., 1997). 5-HT_{2C} receptor agonists such as Ro 60-0175 are also found to reduce operant responding for food on fixed and progressive ratio schedules of reinforcement (Grottick et al., 2000), and on a second-order schedule of reinforcement (Somerville et al., 2007). Our observation that MK212 decreased chocolate pellets hoarding in rats is consistent with these findings, indicating that MK212 has a broad effect on reward (pup or food)-based motivated behaviors. Indeed, 5-HT_{2C} agonists have shown a general inhibitory effect on various motivated behaviors, both natural and drug based (Fletcher et al., 2012; Higgins and Fletcher, 2003; Higgins et al., 2013). However, the magnitude of MK212's disruption appears to be behaviorally selective, as the food retrieval disruption persisted longer than that of pup retrieval: food retrieval disruption occurred at all the testing points, whereas the pup retrieval disruption occurred only at the 30 min point after the drug injection.

In Experiment 1, we found that 6-h food deprivation was unable to reverse MK212-induced deficit on food hoarding (carrying). This may be due to the fact that 6-h food deprivation during the light phase of the diurnal cycle was too weak to activate food motivation. In fact, earlier studies suggest that rats increase their food intake significantly following 2 to 6 hours of food deprivation at night, whereas the same food deprivation during the day cannot induce a change in subsequent food intake (Larue-Achagiotis and Le Magnen, 1982). It is not entirely clear how MK212 might cause a decrease in food motivation. One way is to cause satiation. It is known that activation of 5-HT_{2C} receptors in the arcuate nucleus of the hypothalamus stimulates production of proopiomelanocortin, which is then cleaved into α-melanocyte stimulating hormones. These hormones in turn act on the melanocortin 4 receptor in the paraventricular nucleus of the hypothalamus to induce satiety (Clifton et al.,

2000; Cone, 2005; Heisler et al., 2002; Somerville et al., 2007). MK212 might have this effect on the feeding center of hypothalamus.

As we mentioned, 5-HT_{2C} agonists including MK212 have shown a general inhibitory effect on various motivated behaviors, both natural and drug based (Fletcher et al., 2012; Higgins and Fletcher, 2003; Higgins et al., 2013), it raises a question whether any of MK212's effects is maternally specific. At present, we do not have any evidence to suggest that this is the case. Because 5-HT_{2C} is such an important neuroreceptor for many brain and behavioral functions, even if MK212 has a maternal-specific effect, it is likely got masked by its global effect. The more likely scenario is that MK212 suppresses various psychological functions (e.g. emotion regulation, motivation, motoric response) that underlie various motivated behaviors (including maternal behavior). One important task for future research is to identify the maternal specific action of 5-HT_{2C} receptor and its associated psychological functions.

MK212 binds to 5-HT_{2C} receptors with high affinity, but it also has affinity for other receptors, such as 5-HT_{2A}, 5-HT_{2B} and 5-HT_3 receptors (Cussac et al., 2002; Glennon et al., 1989; Porter et al., 1999). To verify that the effects of MK212 on maternal responses were 5-HT_{2C} receptors mediated, we used a highly selective 5-HT_{2C} receptor antagonist (Kennett et al., 1997), SB242084, and found it effectively reversed the maternal disruptive effects of MK212. Given that SB242084 is a high affinity antagonist for 5-HT_{2C} receptor with 100-fold and 158-fold selectivity over the 5-HT_{2B} and 5-HT_{2A} receptors, respectively (Kennett et al., 1997), and it by itself did not disrupt pup retrieval at 0.2, 0.6 and 1.0 mg/kg (unpublished data), the positive finding of SB242084 in blocking the effects of MK212 suggests that MK212-induced disruption of pup retrieval is mainly mediated by activating specific 5-HT_{2C} receptors. This finding is also in agreement with previous findings showing that central SB242084 injection can block the effects of MK212 on reinstatement of extinguished cocaine-seeking behavior (Pentkowski et al., 2010), and the effect of MK212 on the acquisition of inhibitory avoidance in the elevated T-maze (Yamashita et al., 2011).

Because the NAs and mPFC are densely populated with 5-HT_{2C} receptors (Liu et al., 2007; Pompeiano et al., 1994); lesions of either the NAs or mPFC can cause pup retrieval deficits (Afonso et al., 2007; Li and Fleming, 2003b; Olazabal et al., 2013); and inactivation or inhibition of neuronal activity in the mPFC can disrupt maternal behavior (Febo et al., 2010), we initially thought that microinjection of MK212 into these brain sites would cause a disruption of maternal behavior. This idea was also consistent with our previous c-Fos studies showing that the 5-$HT_{2A/2C}$ agonist DOI and antagonists CLZ and OLZ, though not selective to 5-HT_{2C} receptors, increased c-Fos expression in the NAs and mPFC (Zhao and Li, 2010, 2012). The results here were contradictory to our expectation. In addition, microinjection of MK212 into the MPOA also failed to cause a pup retrieval disruption. There are several possible reasons that may explain these negative findings. First, it is possible that 5-HT_{2C} receptors in these regions are not critical for the expression of maternal behavior. Other neuroreceptors such as dopamine D_1 and D_2 receptors are more important, as direct manipulation of these receptors in the NAs, mPFC and MPOA are shown to alter maternal care in rats (Numan et al., 2005; Numan and Stolzenberg, 2009). Second, the MK212 effect may require a coordinated change in 5-HT_{2C} receptor activity in all these brain regions. Thus single manipulations of individual sites might not be the best way to

capture the systemic effects of MK212. This possibility is consistent with a previous functional magnetic resonance imaging study showing that pup stimuli induce a wide range of brain activation, including olfactory system, NAs, insular cortex, prefrontal cortex, VTA, cortical amygdala, and several cortical and hypothalamic nuclei (Febo et al., 2005). Third, other brain regions might be even more important than the above targeted sites (Barofsky et al., 1983a; Barofsky et al., 1983b). Raphe, as a serotonergic brain region, is important for the regulation of maternal behavior. DR 5-HT neurons project to the hypothalamus, and are involved in the suckling-induced prolactin release; whereas medial raphe (MR) 5-HT neurons directly influence maternal behavior (Barofsky et al., 1983a). It has been shown that MR lesions with 5,7-dihydroxytryptamine (5,7-DHT) cause a higher incidence of abnormal behaviors, including failure to retrieve pups, cannibalism, etc. than sham animals or animals with 5,7-DHT lesions in the DR or superior colliculus. Therefore, 5-HT_{2C} in the MR nucleus may play a more critical role in the mediation of MK212 effects than other regions. This idea needs to be tested in the future study. Finally, the chosen doses of MK212 for central infusions may not be optimal. This possibility appears less likely as intra-mPFC infusion of MK212 in the dose ranges used here inhibits cocaine hyperactivity and attenuates cocaine reinstatement (Filip and Cunningham, 2003; Pentkowski et al., 2010). In addition, intra-NAc MK212 infusions in this dose range also enhance the effect of cocaine cues (Filip and Cunningham, 2002).

To provide a global view of the neural network where MK212 may act to achieve its maternal effect, we used c-Fos immunohistochemistry to map out the possible major sites that MK212 may act on. We observed greater Fos-immunoreactive nuclei in the CeA, and fewer Fos-immunoreactive nuclei in the LSv, MPOA, DG and DR following acute MK212 treatment (Figure 6). In addition, repeated administration of MK212 further decreased c-Fos expression in the DR. These data suggest that the CeA, LSv, MPOA, DG and DR are at least partially involved in the action of MK212. Recent studies show that non-selective 5-HT_{2C} receptor agonists Ro 60-0175 and mCPP inhibit the firing of 5-HT neurons in the DR (Queree et al., 2009), acting via neighboring gamma-aminobutric acid neurons (Liu et al., 2000). Moreover, the serotonergic neurons in the DR project to the VTA and NAc, which may regulate maternal motivation toward the young (Bridges, 2015). Suckling stimuli are also processed through the DR by serotonergic inputs to the NA (Dolen et al., 2013). The CeA appears to be a crucial component for normal activation of maternal aggression circuitry and was shown to mediate the suppression of maternal care (Dulac et al., 2014). Maternal aggression was increased after microinjection of the 5-$HT_{2A/2C}$ receptor agonist α-Methyl-5-hydroxytryptamine maleate into the amygdala (de Almeida et al., 2006). Taken together, we can suggest three neural systems where 5-HT_{2C} receptor agonists such as MK212 may modulate maternal behavior. First, they could affect 5-HT_{2C} receptor activity at the terminal sites of the mesolimbic, mesocortical and niagro-striatal dopamine pathways and modulate the neuronal activity of dopamine neurons and dopamine release (Alex and Pehek, 2007; Bailey et al., 2016). This activity is directly related to appetitive responses and willingness to exert effort toward a goal (Salamone et al., 2007). Second, they could interact with GABAergic and/or glutamatergic systems in the mPFC and regulate impulsivity and motivation (Dalley et al., 2008; Liu et al., 2007; Pentkowski et al., 2010). Finally, they could affect 5-HT_{2C} receptors in the CeA, LSv, MPOA, DG and DR to alter maternal behavior.

Future research needs to be conducted to elucidate the exact central mechanisms of 5-HT_{2C} receptor in maternal behavior.

In conclusion, the present study demonstrated that MK212 disrupts maternal behavior, and this disruption may reflect a general motivational suppressive effect of 5-HT_{2C} agonism or its action on other unidentified processes. Future research is needed to determine the specific neural circuitry through which 5-HT_{2C} receptor agonists produce the inhibitory effects on maternal care.

Acknowledgments

Role of funding source

This research was supported by a grant from the National Institute of Mental Health (5R01MH097718-02). The founding source had no further role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

This research was supported in part by a grant from the National Institute of Mental Health (5R01MH097718-02). We want to thank the anonymous reviewers for their insightful comments which greatly improved the quality of this version of manuscript.

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Highlights

- Pup separation attenuates acute MK212-induced deficit on pup retrieval.
- Acute MK212 inhibits food hoarding.
- SB242084 alleviates MK212-induced maternal disruption.
- Acute MK212 alters c-Fos expression in the LSv, MPOA, DG, DR and CeA.
- Repeated MK212 disrupts pup retrieval persistently and decreases DR activity.

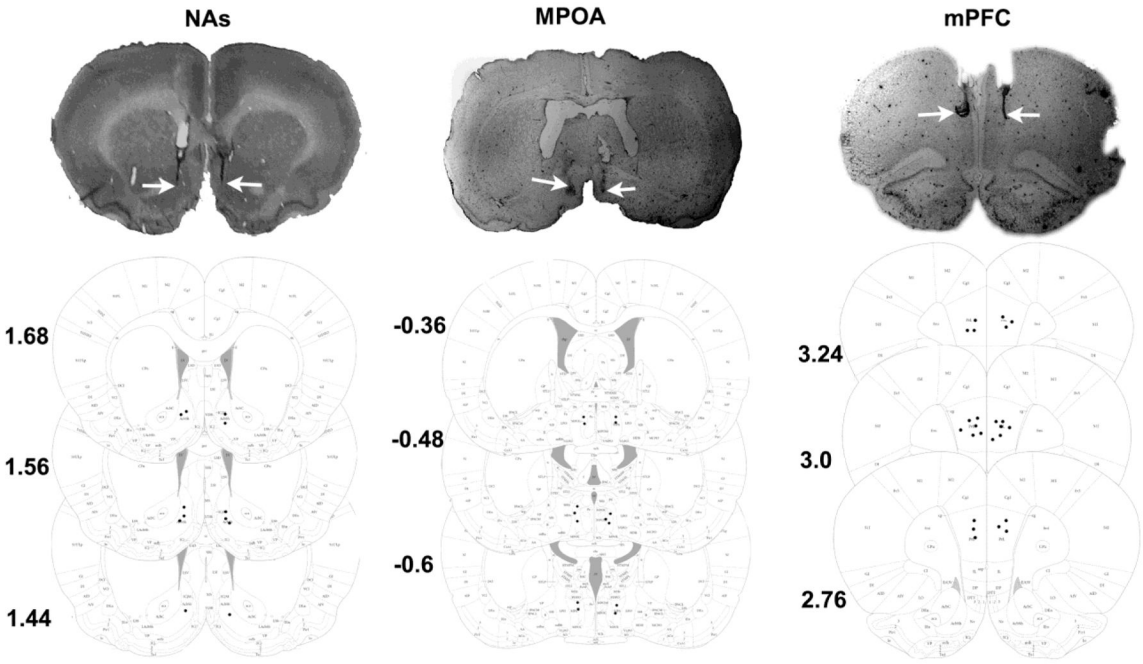

Figure 1.

Histological representations of microinjection sites and schematic diagrams showing the location of the injector tips in the NAs, MPOA and mPFC. Data are reconstructed from Paxinos and Watson (Paxinos, 2005). *Numbers to the left of the sections* indicate anteroposterior distance from bregma in millimeters. The *arrow* in the histological representation section and *black dot* in the schematic diagrams denotes the infusion placement.

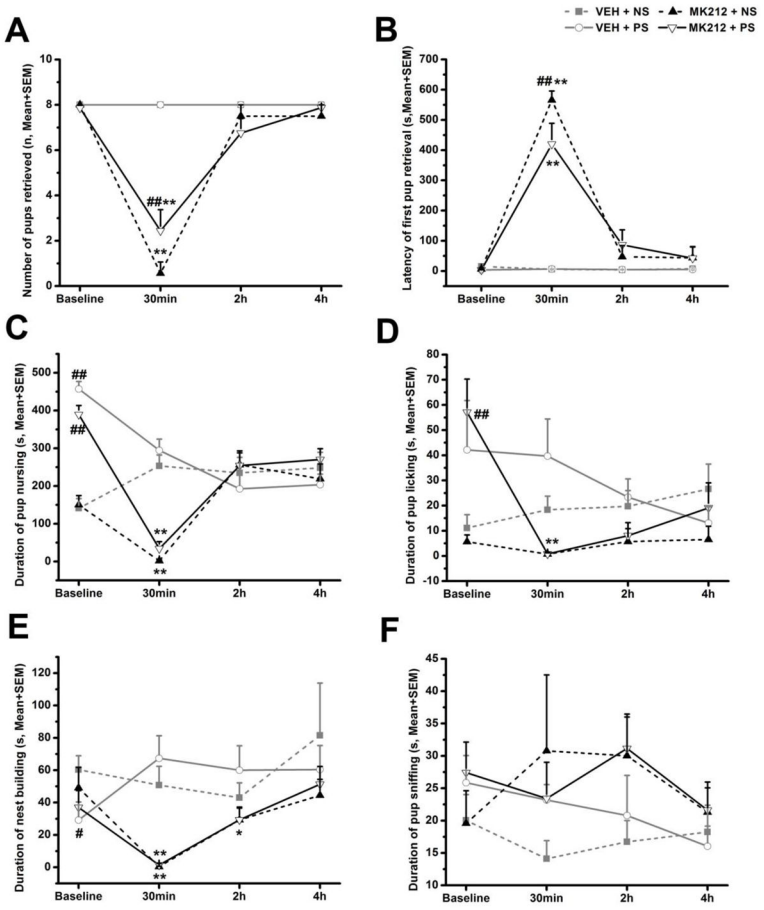

Figure 2.

Effects of systemic MK212 (2.0 mg/kg) on maternal behavior under 4-h pup-separation (PS, pups were taken away from dams 4 h before the test) and no-pup-separation (NS) conditions on PP 4 and 6. On each test day, maternal behavior was observed for 10 min at 4 time points: 0.5 h before, 0.5, 2 and 4 h after the injection. On PP 4, mother rats were tested under two separation conditions. On PP 6, the rats tested in the pup-separation condition on Day 4 were tested in the no-pup-separation condition, and those previously tested in the no-pup-separation condition were tested in the pup-separation condition. Data from the postpartum day 4 and 6 under the same condition (PS or NS) are combined and expressed as mean ± SEM. A, number of pup retrieved; B, latency of first pup retrieved; C, duration of pup nursing; D, duration of pup licking; E, duration of nest building; F, duration of pup sniffing. ** $p < 0.01$, * $p < 0.05$ significantly different between the different drug administrations (VEH vs. MK212) within the same separation condition (PS or NS); ## $p < 0.01$, # $p < 0.05$ significantly different between the different separation conditions (PS vs. NS) within the same drug exposure (VEH or MK212).

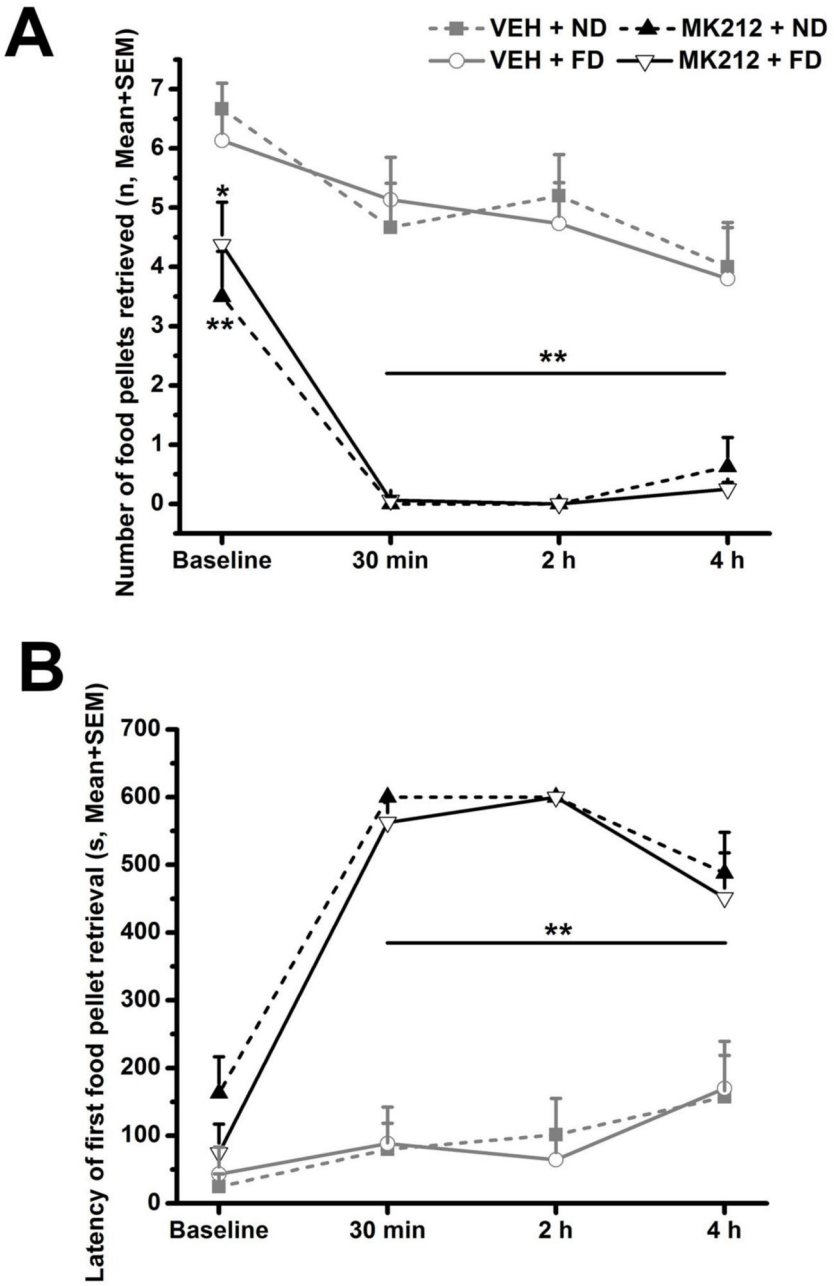

Figure 3.

Effects of systemic MK212 (2.0 mg/kg) on food retrieval under 6-h food-deprivation (FD) and no-food-deprivation (ND) conditions on postpartum day 8 and 10. Each retrieval test lasted 10 min. Number (A) and latency (B) of food retrieval from the postpartum day 8 and 10 under the same condition (FD or ND) are combined and expressed as mean + SEM. * $p < 0.05$, ** $p < 0.01$ significantly different between the different drug administrations (VEH vs. MK212) within the same deprivation treatment (FD or ND).

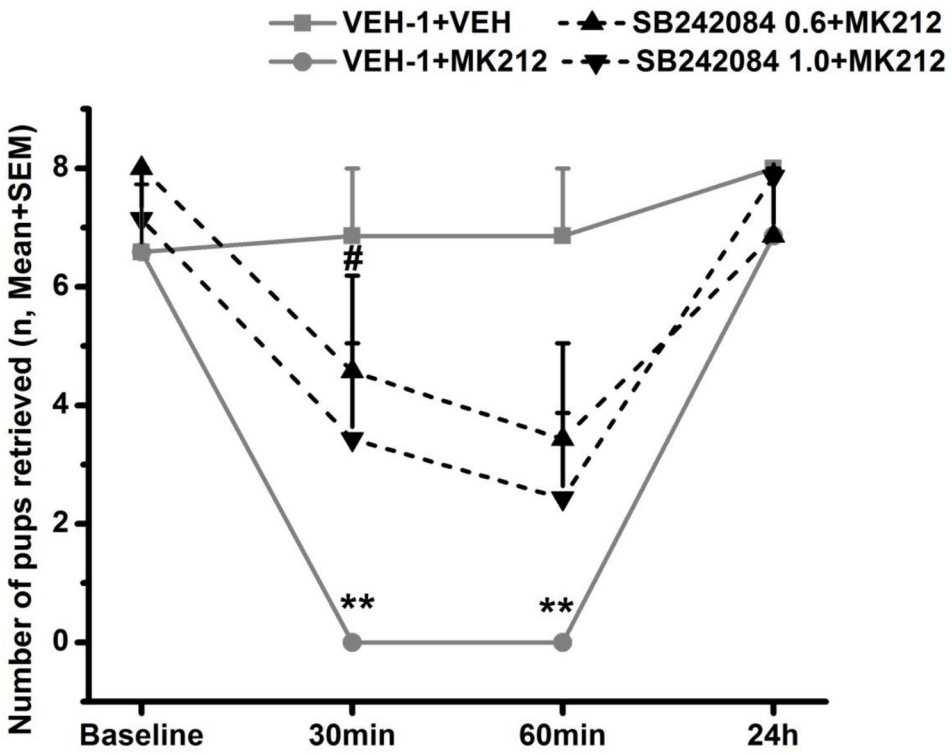

Figure 4.

Effects of acute MK212 (2.0 mg/kg) treatment on pup retrieval with pretreatment of SB242084 (0, 0.6, 1.0 mg/kg). SB242084 was injected 10 min before the MK212. Maternal behavior was tested for 10 min at 30 min before, 30 min, 60 min and 24 h after MK212 injection. Number of pups retrieved in each test is expressed as mean + SEM. ** $p < 0.01$ significantly different between the VEH-1 + VEH and − MK212 groups. # $p < 0.05$ significantly different between SB242084 + MK212 and VEH-1 + MK212 groups.

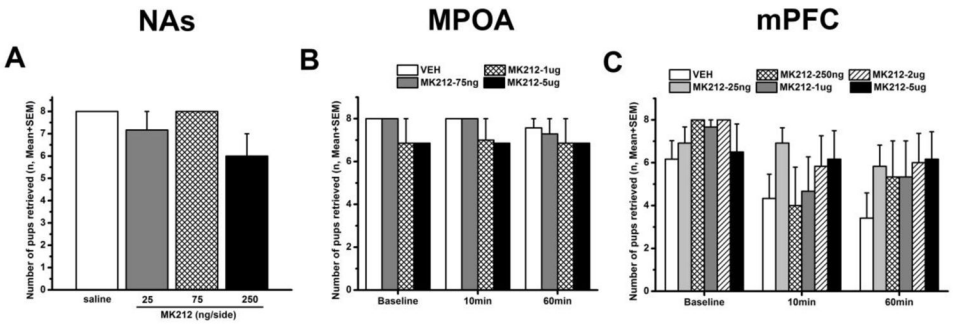

Figure 5.

Effects of MK212 microinfused into the nucleus accumbens shell (NAs, A) on pup retrieval throughout the four test days (PP 3, 5, 7 and 9). Effects of MK212 microinfused into the medial preoptic area (MPOA, B) or medial prefrontal cortex (mPFC, C) on pup retrieval throughout the four test days (PP 4, 6, 8 and 10). Number of pups retrieval in each test is expressed as mean + SEM.

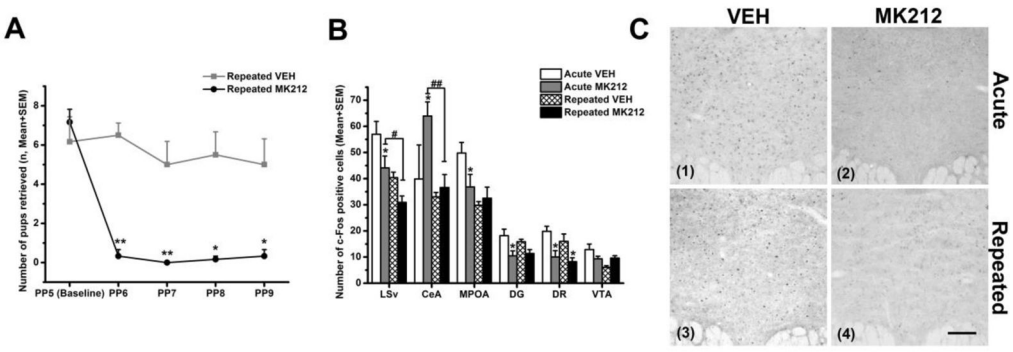

Figure 6.

A c-Fos immunohistochemistry study for the MK212 (2.0mg/kg) effects on maternal behavior. (A) Effects of repeated MK212 administration on the number of pup retrieved. Maternal behavior was tested for 10 min once daily from PP 6 to 9, with starting at 30 min after drug injection. (B) Effects of acute and repeated MK212 administration on c-Fos immunoreactivity. On PP 10, all rats were overdosed and perfused and their brains were extracted for c-Fos immunoreactivity staining 1 h after the drug injection. Number of anti-c-Fos positive cells is expressed as mean + SEM. ** $p < 0.01$, * $p < 0.05$ significantly different between the VEH and MK212 in the same regimen (acute or repeated drug exposure); # $p < 0.05$, ## $p < 0.01$ significantly different between the acute and repeated drug treatment in the same treatment condition (VEH or MK212). LSv, ventral lateral septum; CeA, central amygdala; MPOA, medial preoptic area; DG, dentate gyrus; DR, dorsal raphe; VTA, ventral tegmental area. (C), photomicrographs of immunohistochemistry showing c-Fos expression in the dorsal raphe. In comparison to the VEH groups, MK212 reduced the number of c-Fos immunoreactive neurons under both acute and repeated administration. (1), acute VEH; (2), acute 2.0mg/kg MK212; (3), repeated VEH; (4), repeated 2.0mg/kg MK212. Scale bar =100 μm.